

# Morphological Correlates of the Phonatory Organ in an Ultrasonically Phonating Frog

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**Abstract** The concave-eared torrent frog (*Odorrana tormota*) is the first species of tailless amphibian that was evidenced to phonate and detect ultrasounds. We employed anatomic and histological methods to examine the phonatory organs, including the floor of the buccal cavity, vocal cords and glottis, of *O. tormota* and its sympatric species including *O. graminea*, *O. schmackeri*, and *Amolops wuyiensis* with different fundamental frequencies, and *Pelophylax nigromaculatus* as a control. Our results reveal that *O. tormota* possesses specialized phonatory organ structures, with thinner vocal cords modulated by a moderately stronger muscular mastoideus between the medial vocal cords and the lateral cricoid cartilages, and more elastic mouth floor to likely supply faster air stream which could make the vocal cords vibrate at higher frequencies, larger relative distance between the two muscles m. intermandibulares (RDMI), and higher nucleus density of m. intermandibularis (NDMI) and m. geniohyoideus (NDMG). The results of Pearson's correlation tests between the mean values of the above measurements and the fundamental frequencies from the five species imply that all the specialized phonatory organ structures mentioned above might be favored by higher frequency of phonation of *O. tormota*.

**Keywords** concave-eared torrent frog, comparative anatomy, ultrasonic phonation, fundamental frequency

## 1. Introduction

Sonic communication is one of the most important and common modes of intra-and-inter-species communications in animals. Through this mode of communication, the auditory organs perceive the sound produced by phonatory organs, which can then be interpreted as a message for territorial behavior, courting, alarming, defense and cooperation among individuals (Marler *et al.*, 1977). Therefore, vocal communication is significant to an animal's survival and reproduction (Ryan, 1985). Under natural and sexual selection, different species have evolved different ways for vocal

communication.

Prior to the discovery of the concave-eared torrent frog (*Odorrana tormota*), ultrasonic communication had only been known to exist in a few mammals such as dolphins, whales, bats and some rodents (Feng *et al.*, 2006; Shen, 2006). The concave-eared frog has been studied considerably since it was discovered in China based on its unique concave eardrums, external auditory canal (Wu, 1977) and relatively high frequency of sonic communication (Feng *et al.*, 2006; Shen *et al.*, 2008), which is extremely rare in amphibians (Feng *et al.*, 2006). These ranid frogs live along fast-flowing stream and have adapted their mode of communication to avoid being masked by the wideband background noise of the loud torrential stream which they inhabit (Katti, 2004).

It is well known that structure and function are tightly linked in adaptive evolution. Thus, the vocal organs may display unique characteristics in order to perform ultrasonic phonation. *O. tormota* and another frog, *Huia*

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Received: 16 November 2011 Accepted: 30 July 2012

*cavitympanum* (Boulenger, 1893) from Borneo, were confirmed to communicate with ultrasound (Feng *et al.*, 2006; Arch *et al.*, 2008; Shen *et al.*, 2008; Arch *et al.*, 2009) to screen out the interference of low frequencies of their noisy environment (Suthers *et al.*, 2006). These discoveries inspired the enthusiasm to study the relationship between *O. tormota*'s vocal organs and their related functions.

Frogs phonate mainly with vocal cords of the respiratory system, comprising a pair of elastic fibers attached to the posterior end of the arytenoids (Walkowiak, 2006). Most of the frog's vocal cords are T-shaped, that is, the lateral parts are connected to the laryngeal skeleton and the medial parts of the vocal cords extend cranially and caudally (Walkowiak, 2006). To call, they take up a large volume of air into the mouth cavity and the lungs via several buccal oscillation and lung inflation cycles. Then the air flowing out of the lungs vibrates the vocal cords to make sound (Walkowiak, 2006). The driving force for the inspiratory air stream is generated by the musculature in the mouth floor which was showed to be different among species (Xiong *et al.*, 2010).

In this study, we used anatomical and histological methods to examine the morphometrics of vocal organs, including buccal floor, vocal cords and glottis of *O. tormota* and its sympatric species *O. graminea*, *O. schmackeri*, and *Amolops wuyiensis*, and a common pond frog *Pelophylax nigromaculatus*, and then to elucidate structures required for the derived ultrasonic phonation found in *O. tormota*.

**Table 1** Information of the specimens used in this study.

Species	Fundamental frequency (KHz)		Locality	Specimen No.
<i>O. tormota</i>	$F_{0\min}$	$F_{0\max}$	Huangshan, Anhui, China	20080237-8, 090425001-2, 200805017, 2008050106
	$5.5 \pm 0.8$	$8.3 \pm 1.2$	Jiande, Zhejiang, China	ZJ200806290, ZJ200806293
<i>O. graminea</i>	$3.1 \pm 0.7$	$7.4 \pm 1.1$	Tiantai, Zhejiang, China	ZJ200806078-9, ZJ200806080, ZJ200806262-3
	(Shen <i>et al.</i> , 2011)		Huangshan, Anhui, China	20080322-4, 20080331, 20080328-9, 20080233
<i>O. schmackeri</i>	$2.7 \pm 0.6$	$4.3 \pm 0.4$	Jiande, Zhejiang, China	ZJ200806283, ZJ200806280, ZJ200806285
			Tiantai, Zhejiang, China	ZJ200806074-5, ZJ200806088
<i>A. wuyiensis</i>	$2.2 \pm 0.1$	$2.5 \pm 0.1$	Huangshan, Anhui, China	100722003, 100722005-6
			Jiande, Zhejiang, China	ZJ200806271, ZJ200806268
<i>P. nigromaculatus</i>	$0.5 \pm 0.01$	$0.5 \pm 0.01$	Jiangshan, Zhejiang, China	ZJ200806161-3, ZJ200806159
	(Kuramoto, 1977)		Tiantai, Zhejiang, China	ZJ200806089-90
			Huangshan, Anhui, China	200805039, 20080172, 20080226, 10722007-9, 100722012
			Tiantai, Zhejiang, China	ZJ2009043001, ZJ200806066, ZJ200806068
			Tiantai, Zhejiang, China	ZJ200806031-6, ZJ200806029, ZJ200806030, ZJ200806038

## 2. Materials and Methods

**2.1 Materials** We chose male *O. tormota*, *O. graminea*, *O. schmackeri*, *A. wuyiensis* and *P. nigromaculatus* for this study since under sexual selection the males vocalize to attract females (Wells *et al.*, 2006).

The specimens used in this study were collected in Anhui and Zhejiang, China (Table 1) and deposited at the Chengdu Institute of Biology (CIB), Chinese Academy of Sciences after being fixed in formalin (10%). The skin, m. intermandibularis and m. geniohyoideus (Duellman *et al.*, 1994) were isolated from the mouth floors and fixed in Bouin stationary liquid for more than 12 h in preparation for paraffin histology.

Before collecting specimens, the calls of *O. schmackeri* and *A. wuyiensis* were collected using a Portable Linear PCM Recorder (Sony PCM-D50), and their fundamental frequencies were obtained using the software PRAAT (Boersma *et al.*, 2008). The fundamental frequencies of *O. tormota*, *O. graminea*, and *P. nigromaculatus* were obtained from the literature of Feng *et al.* (2009), Shen *et al.* (2011) and Kuramoto (1977), respectively.

**2.2 Recording and digitizing of calls** Phonations of *O. schmackeri* and *A. wuyiensis* (more than 5 individuals for each species) were recorded and digitized using a Portable Linear PCM Recorder (Sony PCM-D50). The spontaneous phonations were digitized with a sampling rate of 48 kHz and 16-bit accuracy, and stored as individual files. Calls were then categorized according to the degree of frequency modulation and harmonic structure (Feng *et al.*, 2009). The calls of *O. tormota* and *O. graminea* were divided into seven categories and five of them possessed ultrasonic highest harmonics (Feng

*et al.*, 2009; Shen *et al.*, 2011), of which the minimum fundamental frequencies ( $F_{0\min}$ ) and maximum ones ( $F_{0\max}$ ) were retrieved for comparison in this study. Since *O. schmackeri* had a similar spectrogram to the other two *Odorrana* species, the information of fundamental frequencies was extracted in the same way with software PRAAT (Boersma *et al.*, 2008). The  $F_{0\min}$  and  $F_{0\max}$  of *A. wuyiensis* were extracted from its digitized calls, of which the harmonics had small frequency modulation.

**2.3 Dissection of phonation organs and their morphometrics** Vocal cords, arytenoid cartilages, and the organs of the mouth floor including skin and two muscles m. intermandibularis and m. geniohyoideus were dissected and photographed under a stereoscope (Zeiss stemi 2000-C). Skeletons were stained following Chen *et al.* (2002) to be clearly distinguished with muscles.

**Vocal cords measurements:** Since the thin vocal cords could not be measured by vernier caliper conveniently, we took photos of them under a stereoscope (Zeiss stemi 2000-C) and measured the thickness ( $\mu\text{m}$ ) of the middle parts of the vocal cords (TVC) using the graphic analysis software ImageJ (<http://rsbweb.nih.gov/ij/>). Then, the arithmetic mean value was figured out for each species.

**Distance between the two m. intermandibulares:** The distance between right and left m. intermandibulares (Figure 1 A) is related to the settleability of a frog's mouth floor. Larger distance is likely to possess smaller impedance to the descending movements of the mouth floor. Considering this distance would be variable depending on the measuring locations and variable body sizes, we conducted the measurement at the middle level of lengthways axis of the frog's ventral head and standardized the distance by being divided by the head breadth (W in Figure 1 A) at the same level to get a rate (a/W, i. e., RDMI).

**Width of m. geniohyoideus:** The total width of right and left m. geniohyoideus (Figure 1 B) is related to the settleability of a frog's mouth floor. Smaller width may possess smaller impedance to the descending movement of the mouth floor. Considering this width would be variable depending on the measuring locations and variant body sizes, we took the measurement at the middle level of lengthways axis of the frog's ventral head and standardized them by dividing the head breadth (Figure 1 B) at the same level to get a rate [ (b + c)/W, i. e., RWMG].

**2.4 Buccal organ histology and morphometrics** The skin, m. intermandibularis and m. geniohyoideus were embedded in paraffin using standard methods (Wang *et al.*,

2007), and were then cut into sections of 8–10  $\mu\text{m}$  in thickness.

**Measurement of the thickness of skins:** Skin is the first layer of the mouth floor of a frog, its thickness might be related to the buccal movements, so we measured this characteristic accurately by preparing transverse paraffin sections of the skin which were then analyzed under a microscope (Nikon, Eclipse 55i), with the best four slices selected for photographing. Then, the thickness ( $\mu\text{m}$ ) of skin (including epidermis and dermis) was measured on the photos by the software ImageJ. The arithmetic mean value was calculated for each specimen based on the data from the four slices mentioned above.

**Measurement of the nucleus densities of m. intermandibularis and m. geniohyoideus:** Muscles are comprised of thousands of myocytes containing myofibrils, which are long chains of sarcomeres, the contractile units of the muscle cells. Striated/skeletal muscle cells have multiple nuclei per cell (Miller *et al.*, 2002) which are called syncytia. Usually, nuclei locate where the syncytium shows the highest activity, so the nucleus density of the syncytium might be positively related to its activity and the nucleus density of the syncytium of buccal muscles from ultrasonic frogs might be higher than the one from frogs with low-frequency phonation. To test that, the longitudinal paraffin sections of the muscles were observed under a microscope (Nikon, Eclipse 55i), and of them, the best three slices were selected for photographing. On each photo, the numbers of nuclei from three areas were totaled up to calculate the nucleus density (Nuclei/1000  $\mu\text{m}^2$ ). Then, the arithmetic mean value was calculated for each specimen based on the data from the three slices mentioned above.

## 2.5 Statistical analyses

**Analysis of morphological data:** We used the software OriginPro 8 (<http://www.originlab.com>) to test for differences among the species' means for each measured parameter using a One-way ANOVA, and performed pairwise comparisons with a Tukey's post-hoc test.

**Mean cross-correlations between the six measurements and the fundamental frequencies from the five species:** We took linear regression simulations to show the correlations between the five species' fundamental frequencies (minimum and maximum) and the thickness of the vocal cords, thickness of the skin, relative distance between the two m. intermandibulares, relative width of m. geniohyoideus, and nucleus density of m. intermandibularis and the m. geniohyoideus, respectively. Then, the Pearson correlation tests imbedded in OriginPro 8 were taken to explore the significances of the

correlations. We also took linear regression simulations and Pearson correlation tests based on the data from the three species of the genus *Odorrana* considering their similar genetic background.

### 3. Results

**3.1 Fundamental frequencies** *Odorrana tormota* had the highest  $F_{0\min}$  and  $F_{0\max}$ , followed by *O. graminea*, *O. schmackeri* and *A. wuyiensis* (Table 1). *P. nigromaculatus*' fundamental frequency was shown to be approximately 0.5 kHz (Kuramoto, 1977), which was lower than that of the other four species.

**3.2 Larynx comparison** Comparison between the larynxes of the five species showed that *O. tormota* and *O. graminea* had larger muscles to cover the arytenoid cartilages, which possessed more obvious indentations on the contact surfaces. Resembling the description by (Schmid, 1976), all five species in this study had T-shaped vocal cords (Figure 2), that is, the medial parts of the vocal cords (Figure 2 A) extend cranially and caudally, and the lateral parts (Figure 2 B) are connected to the laryngeal skeleton. The lateral parts of *O. tormota* connect the middle parts tightly but loosely in *P. nigromaculatus* (Figure 2 E). In addition, the medial and lateral parts of vocal cords integrated together in *A. wuyiensis*.

*Odorrana tormota* had the thinnest vocal cords ( $72.04 \pm 20.61 \mu\text{m}$ ,  $n = 9$ ), followed by *O. schmackeri* ( $148.05 \pm 31.57 \mu\text{m}$ ,  $n = 8$ ), *A. wuyiensis* ( $162.27 \pm 55.90 \mu\text{m}$ ,  $n = 9$ ), *O. graminea* ( $174.94 \pm 43.28 \mu\text{m}$ ,  $n = 9$ ) and *P. nigromaculatus* ( $331.86 \pm 49.18 \mu\text{m}$ ,  $n = 9$ ). The data sets from the five species showed significant difference (One-way ANOVA:  $F_{4,39} = 42.3$ ,  $P < 0.05$ ). The Tukey test showed that the thickness of vocal cords in *P. nigromaculatus* was significantly different from that of the other species ( $P < 0.05$ ), and the same as that of *O. tormota*. The remaining three species did not show significant difference from each other (Table 2).

**3.3 Relative distance between the two m. intermandibulares (RDMI)** *Odorrana tormota* had the largest RDMI ( $0.2764 \pm 0.0477$ ,  $n = 8$ ), followed by *O. graminea* ( $0.2595 \pm 0.0352$ ,  $n = 9$ ), *O. schmackeri* ( $0.2130 \pm 0.0616$ ,  $n = 9$ ), *A. wuyiensis* ( $0.1950 \pm 0.0465$ ,  $n = 9$ ) and *P. nigromaculatus* ( $0.0294 \pm 0.0266$ ,  $n = 7$ ). The data sets from the five species showed significant difference (One-way ANOVA:  $F_{4,37} = 33.7$ ,  $P < 0.05$ ). The Tukey test showed there was no significant difference between *O. tormota* and *O. graminea*, between *O. tormota* and *O. schmackeri*, between *O. graminea* and *O. schmackeri*, and between *O. schmackeri* and *A. wuyiensis*, while the rest pair-wise comparisons showed significant ( $P < 0.05$ ) or extremely significant ( $P < 0.01$ ) differences (Table 2).

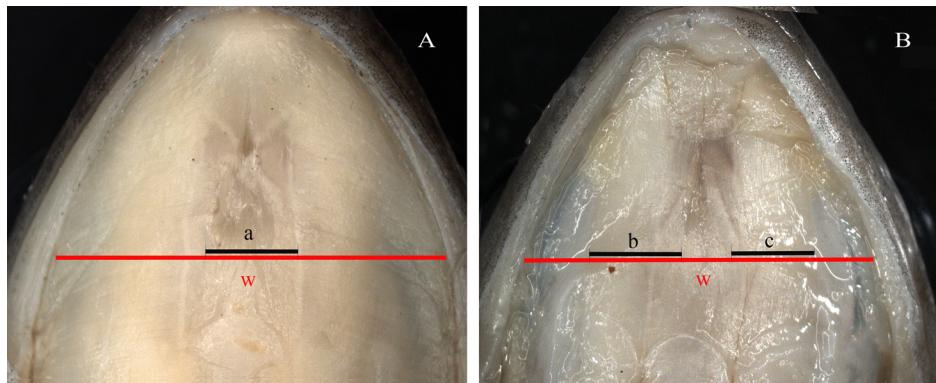
**3.4 Relative width of m. geniohyoideus (RWMG)** *Pelophylax nigromaculatus* had the biggest RWMG ( $0.5591 \pm 0.0862$ ,  $n = 7$ ), followed by *O. schmackeri* ( $0.4977 \pm 0.0367$ ,  $n = 8$ ), *O. graminea* ( $0.4151 \pm 0.0383$ ,  $n = 8$ ), *A. wuyiensis* ( $0.3856 \pm 0.1143$ ,  $n = 8$ ) and *O. tormota* ( $0.3422 \pm 0.0497$ ,  $n = 8$ ). The data sets from the five species showed significant difference (One-way ANOVA:  $F_{4,34} = 11.4$ ,  $P < 0.05$ ). The Tukey test showed there was no significant difference between *O. tormota* and *O. graminea*, between *O. tormota* and *A. wuyiensis*, between *O. graminea* and *O. schmackeri*, between *O. graminea* and *A. wuyiensis* and between *O. schmackeri* and *P. nigromaculatus*, while the rest pairwise comparisons showed significant ( $P < 0.05$ ) or extremely significant ( $P < 0.01$ ) differences (Table 2).

**3.5 Comparison of the thicknesses of the skin from the buccal floor (TSMF)** The skins of the five species were typically composed of two primary layers: the epidermis (Figure 3 C) and the dermis (Figure 3 E), where granular glands and mucus glands are scattered.

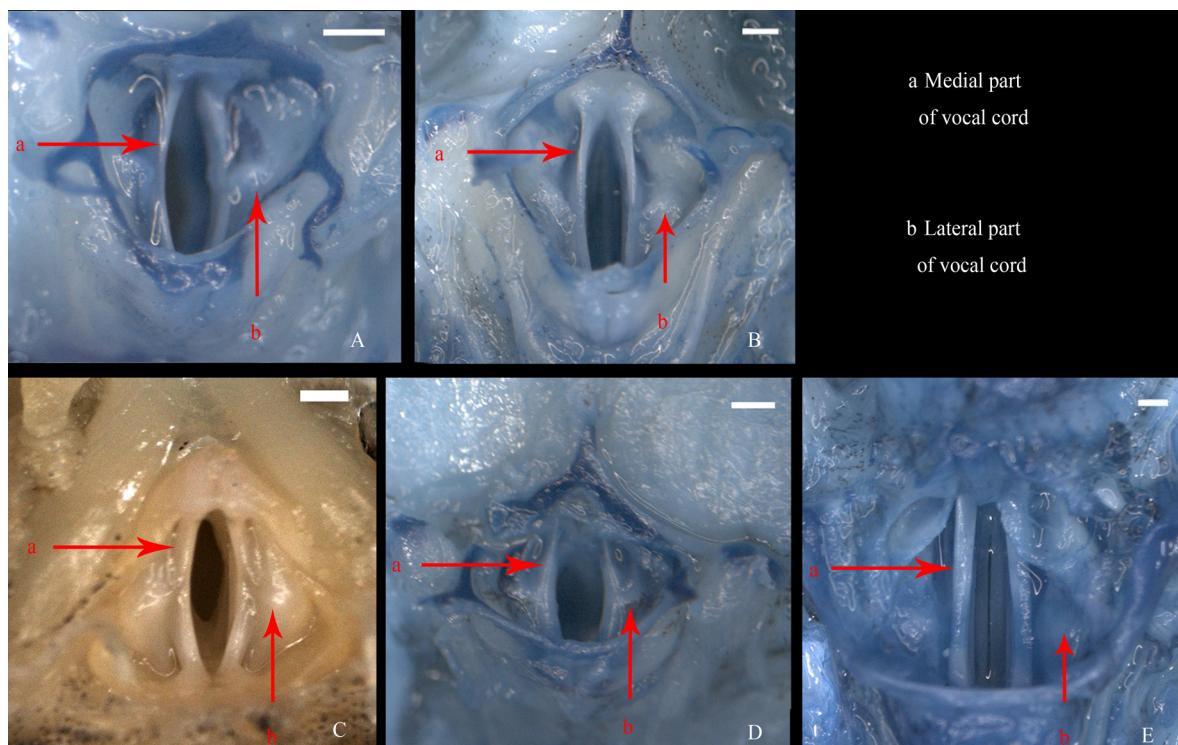
The torrent species, *O. tormota* ( $90.05 \pm 19.55 \mu\text{m}$ ,  $n = 11$ ), *O. graminea* ( $204.88 \pm 56.04 \mu\text{m}$ ,  $n = 12$ ), *O. schmackeri* ( $164.71 \pm 70.95 \mu\text{m}$ ,  $n = 10$ ) and *A. wuyiensis*

**Table 2** The  $P$  values in Tukey tests for pair-wise comparisons of the six measurements of the five species.

Comparison	TVC	TSMF	RDMI	RWMG	NDMI	NDMG
<i>O. tormota</i> vs. <i>O. graminea</i>	0.0001	0.0001	0.9407	0.2642	0.5611	0.0006
<i>O. tormota</i> vs. <i>O. schmackeri</i>	0.0057	0.0218	0.0523	0.0010	0.1219	0.0000
<i>O. tormota</i> vs. <i>A. wuyiensis</i>	0.0005	0.6904	0.0068	0.7386	0.0182	0.0000
<i>O. tormota</i> vs. <i>P. nigromaculatus</i>	0.0000	0.0000	0.0000	0.0000	0.0002	0.0000
<i>O. graminea</i> vs. <i>O. schmackeri</i>	0.6875	0.4216	0.2203	0.1620	0.8635	0.0678
<i>O. graminea</i> vs. <i>A. wuyiensis</i>	0.9683	0.0059	0.0380	0.9197	0.3564	0.6656
<i>O. graminea</i> vs. <i>P. nigromaculatus</i>	0.0000	0.6929	0.0000	0.0035	0.0096	0.0163
<i>O. schmackeri</i> vs. <i>A. wuyiensis</i>	0.9570	0.3757	0.9198	0.0260	0.8908	0.4797
<i>O. schmackeri</i> vs. <i>P. nigromaculatus</i>	0.0000	0.0465	0.0000	0.4645	0.0961	0.9746
<i>A. wuyiensis</i> vs. <i>P. nigromaculatus</i>	0.0000	0.0003	0.0000	0.0004	0.4964	0.1662



**Figure 1** Diagrams to show how to measure the relative distance between two m. intermandibulares (A) and the relative width of m. geniohyoideus (B). a: Distance between right and left m. intermandibulares; b, c: Width of right and left m. geniohyoideus; W: Head width at the middle level of lengthways axis of the frog's ventral head.

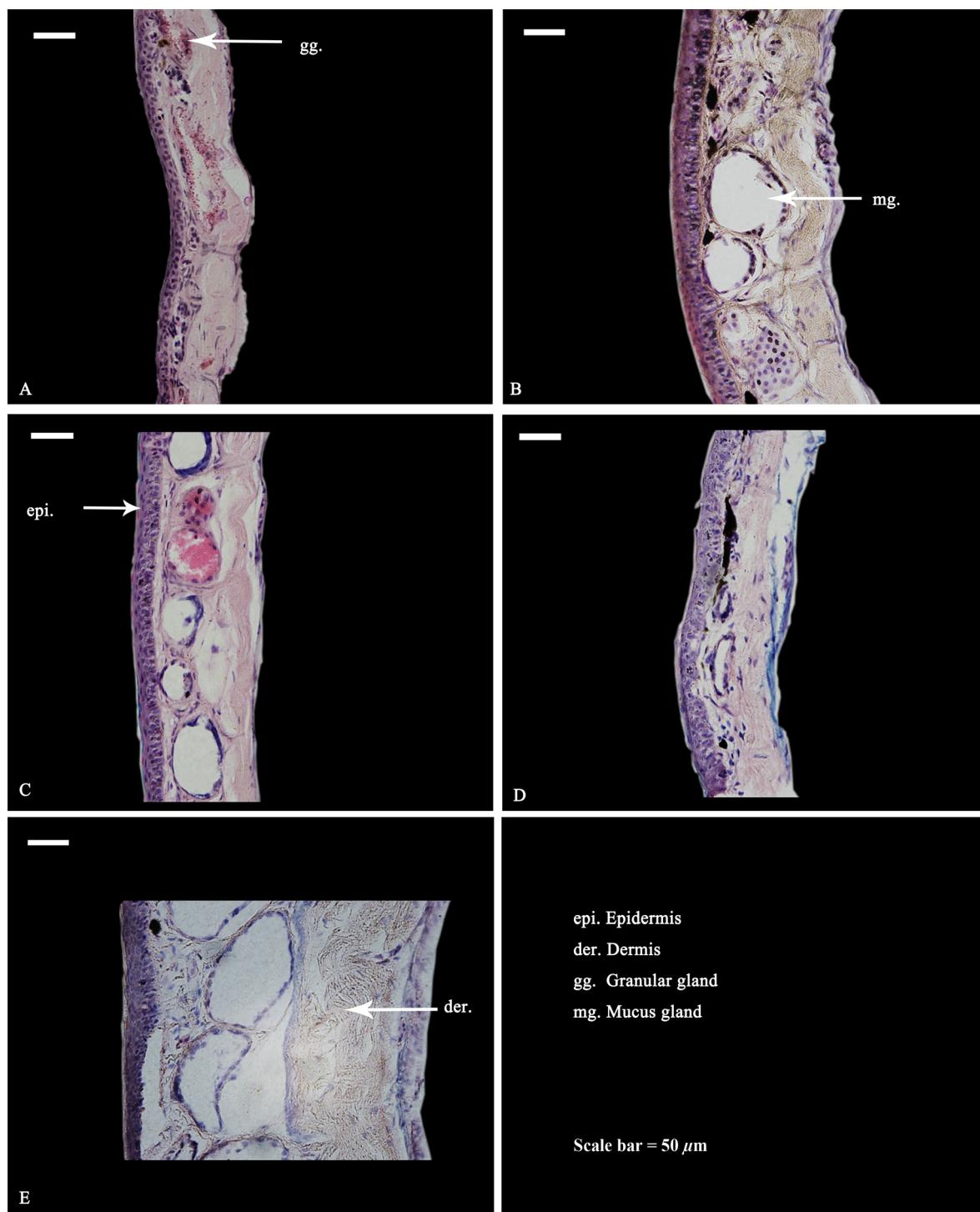


**Figure 2** Vocal cords of the five species studied in this work. A: *O. tormota*; B: *O. graminea*; C: *O. schmackeri*; D: *A. wuyiensis*; E: *P. nigromaculatus*. Scale bar = 500  $\mu$ m.

$(120.81 \pm 36.54 \mu\text{m}, n = 10)$  had thinner skin than the lentic pond frog *P. nigromaculatus*  $(235.82 \pm 71.90 \mu\text{m}, n = 9)$ . Significant difference was found among them (One-way ANOVA:  $F_{4,47} = 12.4, P < 0.05$ ), while there was no significant difference between *O. tormota* and *A. wuyiensis*, between *O. graminea* and *O. schmackeri*, between *O. graminea* and *P. nigromaculatus*, and between *A. wuyiensis* and *O. schmackeri*, but the remaining pairwise comparisons showed significant ( $P < 0.05$ ) or extremely significant ( $P < 0.01$ ) differences.

### 3.6 Cell nucleus density of m. intermandibularis

**(NDMI)** The oval-shaped cell nuclei could be seen from the longitudinal sections (Figure 4) of the m. intermandibularis under the microscope (Nikon, Eclipse 55i). The nucleus densities of m. intermandibularis from the five species showed significant difference (One-way ANOVA:  $F_{4,37} = 6.9, P < 0.05$ ). The lentic-breeding frog *P. nigromaculatus*' density  $(0.96 \pm 0.15 \text{ nuclei}/1000 \mu\text{m}^2, n = 8)$  was lower than that of the lotic-breeding frogs, among which *O. tormota*  $(2.34 \pm 0.40 \text{ nuclei}/1000 \mu\text{m}^2, n = 8)$  was higher than *O. graminea*  $(1.93 \pm 0.90 \text{ nuclei}/1000 \mu\text{m}^2, n = 9)$ , *O. schmackeri*  $(1.67 \pm 0.53 \text{ nuclei}/1000 \mu\text{m}^2, n = 9)$  and *A. wuyiensis*  $(1.42 \pm 0.51 \text{ nuclei}/1000 \mu\text{m}^2, n = 8)$ .

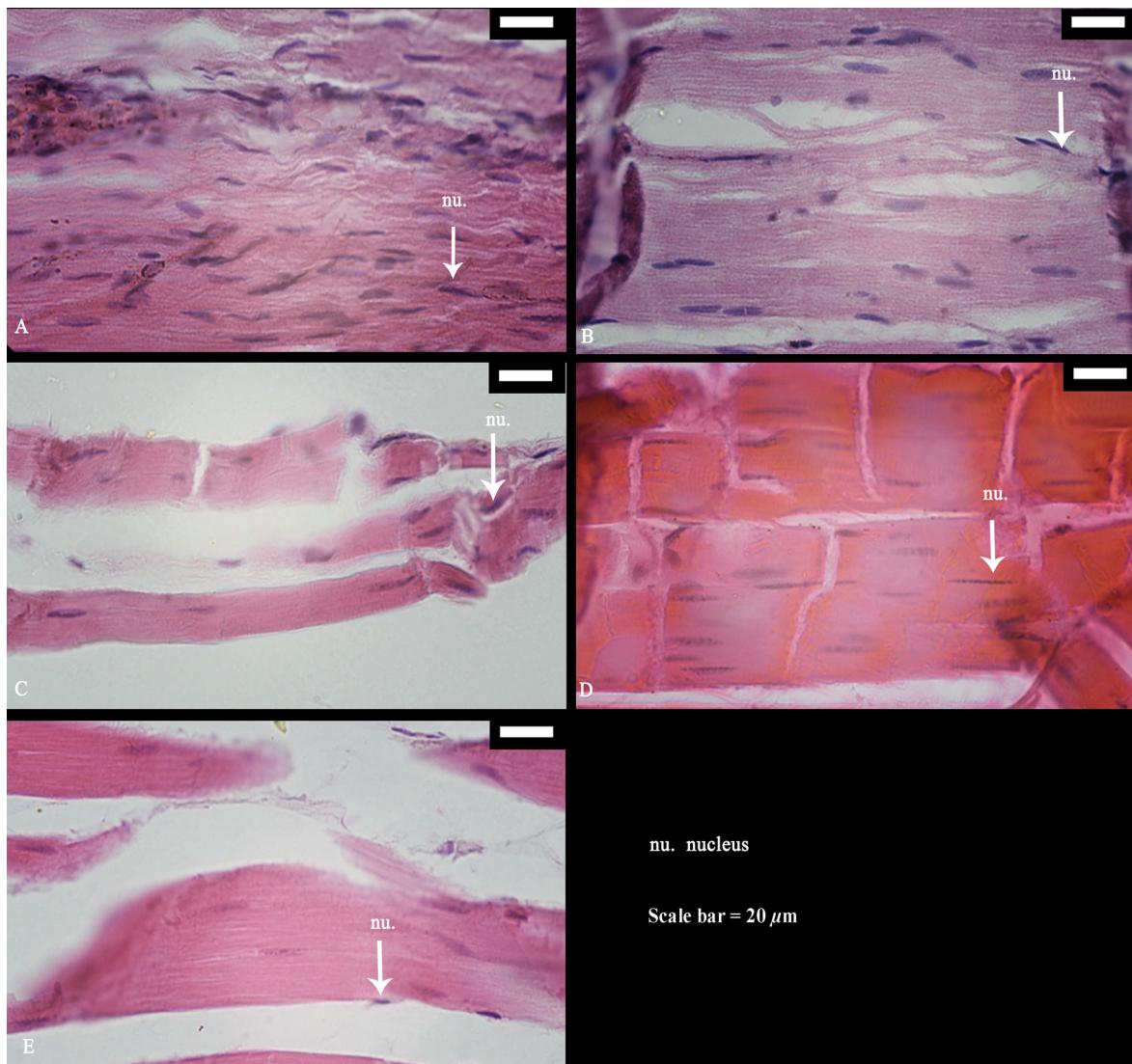


**Figure 3** Transverse sections of skin of the mouth floor from *O. tormota* (A), *O. graminea* (B), *O. schmackeri* (C), *A. wuyiensis* (D) and *P. nigromaculatus* (E).

nuclei/1000  $\mu\text{m}^2$ ,  $n = 8$ ). The Tukey test showed there were significant differences between *O. tormota* and *A. wuyiensis*, between *O. tormota* and *P. nigromaculatus* and between *O. graminea* and *P. nigromaculatus*. However, the rest pair-wise comparisons showed no significant ( $P < 0.05$ ) or extremely significant ( $P < 0.01$ ) interspecific differences.

### 3.7 Cell nucleus density of m. geniohyoideus (NDMG)

The nucleus densities of m. geniohyoideus from the five species showed significant differences (One-way ANOVA:  $F_{4, 29} = 23.1$ ,  $P < 0.05$ ). The ultrasonic frog *O. tormota*'s density ( $1.89 \pm 0.29$  nuclei/1000  $\mu\text{m}^2$ ,  $n = 6$ ) was the highest, and *P. nigromaculatus*' ( $0.88 \pm 0.18$  nuclei/1000  $\mu\text{m}^2$ ,  $n = 8$ ) was the lowest, between which



**Figure 4** Longitudinal sections of *m. intermandibularis* of *O. tormota* (A), *O. graminea* (B), *O. schmackeri* (C), *A. wuyiensis* (D) and *P. nigromaculatus* (E).

*O. graminea* ( $1.29 \pm 0.24$  nuclei/ $1000 \mu\text{m}^2$ ,  $n = 5$ ), *O. schmackeri* ( $0.95 \pm 0.17$  nuclei/ $1000 \mu\text{m}^2$ ,  $n = 7$ ) and *A. wuyiensis* ( $1.13 \pm 0.20$  nuclei/ $1000 \mu\text{m}^2$ ,  $n = 8$ ) were placed. The Tukey test showed there was no significant difference between *O. graminea* and *O. schmackeri*, between *O. graminea* and *A. wuyiensis*, between *O. schmackeri* and *A. wuyiensis*, between *O. schmackeri* and *P. nigromaculatus* and between *A. wuyiensis* and *P. nigromaculatus*. However, the rest pair-wise comparisons showed significant ( $P < 0.05$ ) or extremely significant ( $P < 0.01$ ) interspecific differences.

**3.8 Mean cross-correlations between the six measurements and fundamental frequencies from the five species** The linear regression simulation showed that both the  $F_{0\text{min}}$  and  $F_{0\text{max}}$  correlated positively with RDMI,

NDMI and NDMG, negatively with TVC, TSMF and RWMG either among all the five species or only the three *Odorrana* species in this study (Figure 5; Figure 6).

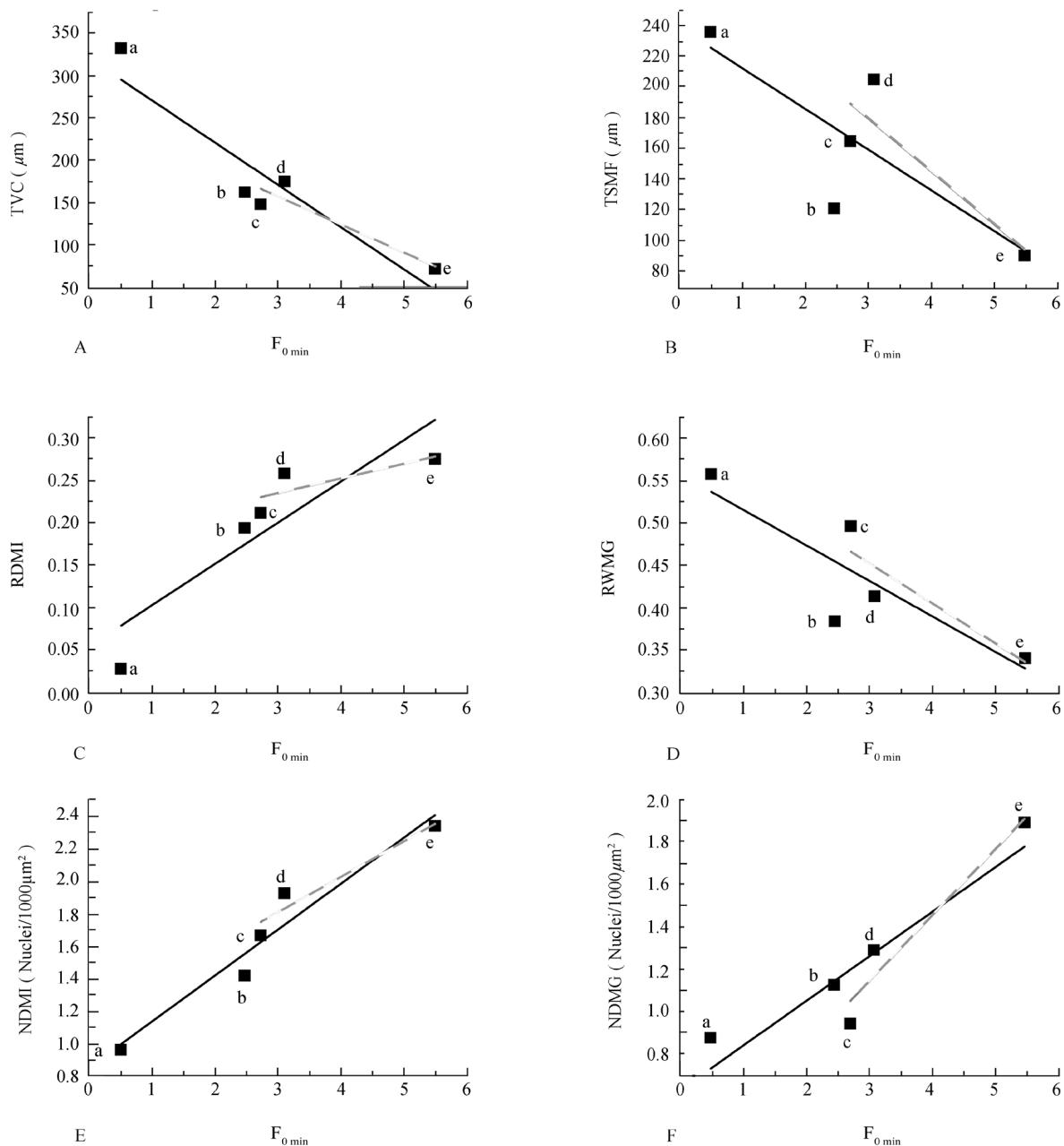
The Pearson correlation tests (Table 3) showed that the  $F_{0\text{min}}$  was significantly correlated with TVC, RDMI, NDMI and NDMG among the five species ( $P < 0.05$ ) while not significantly correlated with any of the six measurements among the three *Odorrana* species ( $P > 0.05$ ); the  $F_{0\text{max}}$  was significantly correlated with RDMI ( $P < 0.05$ ) either among the five species or among the three *Odorrana* species, while significantly correlated with NDMI ( $P < 0.05$ ) only found among the five species.

## 4. Discussion

**4.1 Frog call frequencies** In this study, we focused on

the fundamental frequencies, including the minimum and maximum ones, to explore the relationship between the frequencies of frog calls and the morphometrics mentioned above. According to the source-filter theory of voice production (Fant, 1960), the spectral structure of phonations results from the combination of producing sound at a sound source and the subsequent filtering of the sound in the vocal tract (Feng *et al.*, 2009). This mechanism implies that the fundamental frequency ( $F_0$ ), as the frequency of the first harmonic of most frog calls

is determined by the vocal cords, and then the resonance frequencies (associated harmonics) are provided by the supra-laryngeal vocal tract which can extend sounds to get a maximum frequency at the level of the highest harmonic. As our recorder had a recording limit within frequency (< 20kHz), we could not test the correlations between the maximum frequencies and the six morphometrics directly, but the retrieved data (Kuramoto, 1977; Feng *et al.*, 2009; Shen *et al.*, 2011) did show the positive correlation between the fundamental frequencies



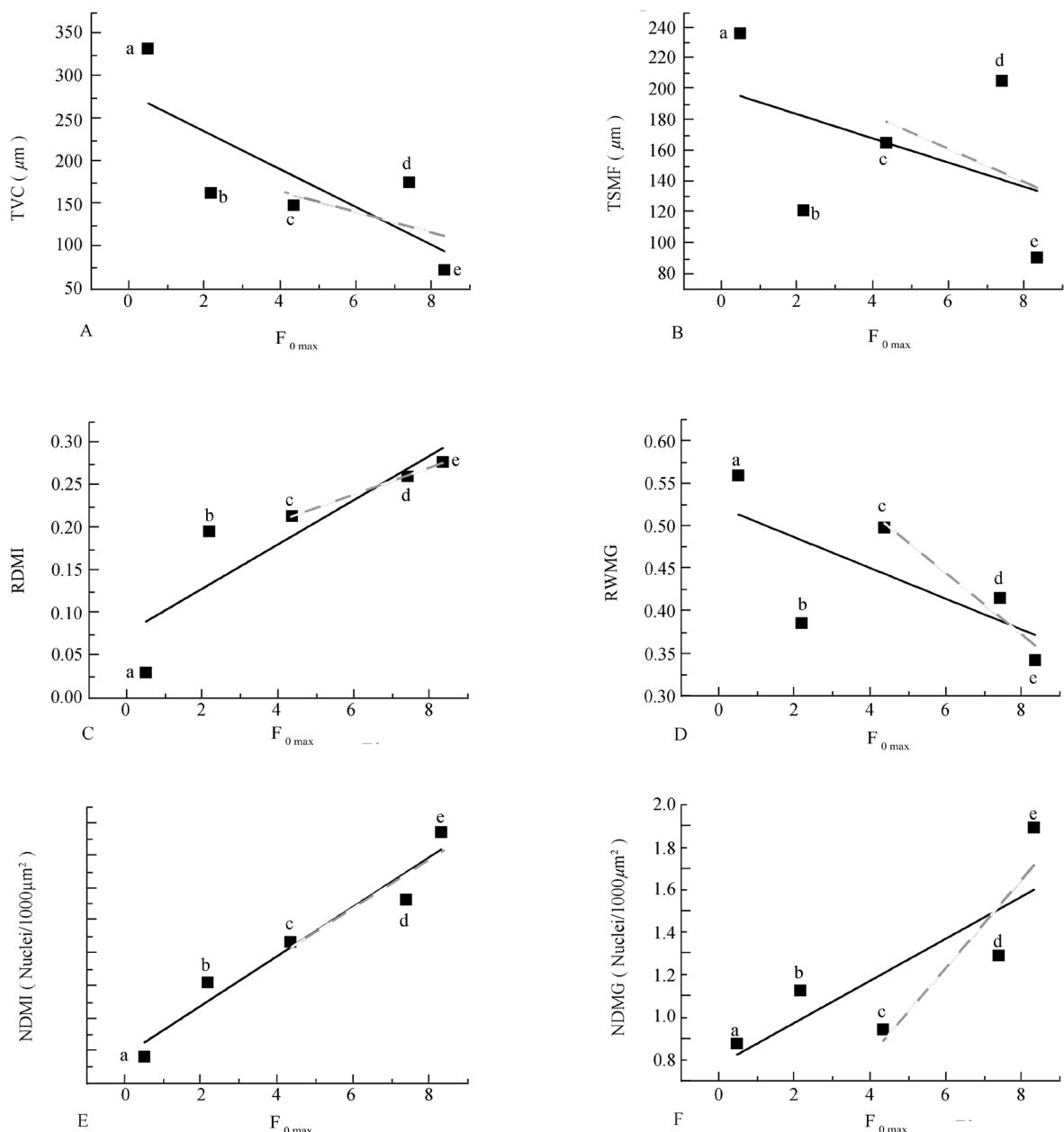
**Figure 5** Linear regression simulations to show the relationship between  $F_0$  min and TVC (A), TSMF (B), RDMI (C), RWMG (D), NDMI (E) and NDMG (F) among all the five species (black line) or the three *Odorrana* species (grey dash line). a: *O. tormota*; b: *O. graminea*; c: *O. schmackeri*; d: *A. wuyiensis*; e: *O. nigromaculatus*.

and the maximum frequencies, namely, higher maximum frequencies were likely to accompany higher fundamental frequencies. Furthermore, the fundamental frequencies of the five species here were also their dominant frequencies (Kuramoto, 1977; Feng *et al.*, 2009; Shen *et al.*, 2011), which are defined to be the frequency occurring most often in a spectrogram with the highest power distribution. So the fundamental frequency could be a perfect representative characteristic of spectrogram to probe into the relationships between the call's frequencies and the phonatory organs, especially vocal cords (the

sound source organs) and mouth floor which originally power the vibration of vocal cords.

#### 4.2 Features of phonatory organs of *O. tormota*

Results from the Pearson correlation tests imply that understanding the mechanism of high frequency phonation of frogs should focus on two key structures: 1) the vocal cords which are the main source of phonation; and 2) the mouth floor which as a “pump” supplies air stream to oscillate the vocal cords. Thus, the thinner vocal cords, the larger RDMI, and the higher NDMI and NDMG might be superior for the higher frequency



**Figure 6** Linear regression simulations to show the relationship between  $F_{0 \text{ max}}$  and TVC (A), TSMF (B), RDMI (C), RWMG (D), NDMI (E) and NDMG (F) among all the five species (black line) or the three *Odorrana* species (grey dash line). a, b, c, d, and e indicating the same species as in Figure 5.

**Table 3** The mean cross-correlation coefficients ( $r$ ) and the significances (Sig.) in the Pearson correlations between the measurements in anatomy and histology and the  $F_{0\min}$  and  $F_{0\max}$  among all the five species or the three species of *Odorrana* in this study, respectively.

			TVC	TSMF	RDMI	RWMG	NDMI	NDMG
5 species	$F_{0\min}$	$r$	-0.93359	-0.79498	0.88249	-0.85032	0.96998	0.92169
		Sig.	0.02034	0.10795	0.04749	0.06793	0.00621	0.02600
	$F_{0\max}$	$r$	-0.78035	-0.44188	0.88440	-0.68887	0.97108	0.81343
		Sig.	0.11942	0.45627	0.04635	0.19831	0.00588	0.09398
3 species of <i>Odorrana</i>	$F_{0\min}$	$r$	-0.92839	-0.88790	0.78947	-0.90752	0.96484	0.97039
		Sig.	0.24239	0.30433	0.42071	0.27594	0.16931	0.15531
	$F_{0\max}$	$r$	-0.4738	-0.38601	0.99937	-0.96561	0.90876	0.89936
		Sig.	0.68577	0.74771	0.02266	0.16743	0.27406	0.28806

phonation of *O. tormota*.

Compared to *O. graminea*, *O. schmackeri*, *A. wuyiensis* and *P. nigromaculatus*, *O. tormota* was shown to possess a more mobilizable hyoid, of which the anterior hyale was composed by two curves with larger bending (Xiong *et al.*, 2010). The hyoid plate of *O. tormota* was concave and was at a higher level than the mandible, which was different to *O. graminea*, *O. schmackeri* and *A. wuyiensis* whose hyoid plate and mandible were at the same level. In addition, *P. nigromaculatus* extruded the hyoid plate outward to make it lower than the mandible.

**4.3 Respiration and phonation of frogs** Call generation of most frogs is closely tied to their buccopharyngeal respirations (Walkowiak, 2006). In buccopharyngeal respiration, air is exchanged between the buccal cavity and the lungs through rapid pulses in the throat. Firstly air is sucked in through the nostrils to fill the buccopharyngeal cavity, and then the expanded mouth floor contracts. This forces the air from the buccopharyngeal cavity into the lungs. To call, they take up a large volume of air into the mouth cavity and lungs via several buccal oscillation and lung inflation cycles, and then the air is expelled out to vibrate the vocal cords for sound producing. Exchange of fresh air in the buccal cavity is managed by periodically elevating and lowering the mouth floor when the nares are open. So, the muscles and bones of the mouth floor coordinate as a pump to produce the air stream during the phonation.

Normally, frogs produce sound with their vocal cords, so the phonation frequencies of a frog mainly depend on the vibration frequencies of its vocal cords. During sound production, the air stream out of the lungs drives the vocal cords vibrating at certain frequencies. The vibration frequencies of vocal cords are negatively related to their thickness and positively to the velocity of the air stream to a great extent, in theory, faster stream would drive the thinner vocal cords vibrating at higher frequencies. To make the fast stream, the air pressure should be largely

different between the lungs and the buccal cavity. So, the phonation features of a frog would depend on the physical characteristics of the vocal cords and the pressure difference driving air stream pass the vocal cords, which is further decided by the biophysics characteristics of the mouth floor.

To sum up, frogs may phonate sound at a high frequency under 3 conditions: 1) fast air stream to power the vocal cords' oscillation; 2) thin vocal cords; and 3) relatively small amplitudes of the vocal cords to improve the vibration times per unit time.

**4.4 Mechanism for *O. tormota*'s phonating at high frequencies** Through the comparative analysis, we found that *O. tormota* could meet the above conditions in principle; it possesses the reasonable structure for high frequency phonation.

**4.4.1 Fast air stream driving vocal cords to vibrate** To produce a fast air stream passing through the vocal cords, a frog needs two basic features: on the one hand, it needs great power to pump much more air into the lungs for creating large pressure difference between the two sides of glottis, this rests with the contractility of the mouth floor, and on the other hand it needs special glottis structures to regulate the passing stream for driving vocal cords to vibrate at a high frequency.

**Higher contractility of the mouth floor:** Since *O. tormota* can phonate at high frequencies, there should be an acoustic source (i. e., the vocal cords) that can oscillate with high frequency driven by the fast air stream. So it should have mouth floor with strong contractility to make sure that a large volume of air could be pumped into its lungs.

In this study, the comparative anatomy of the five species showed that *O. tormota* had mouth floor with the highest contractility potentially. One side, it had looser skin, a concave hyoid plate (Xiong *et al.*, 2010), and the largest relative distance between m. intermandibulares and thinner and narrower m. geniohyoideus, might

contributing to the high settleability of the mouth floor, which could enlarge the volume of the buccal cavity to inhale more air.

Another side, it was inferred to possess greater contractibility as a powerful air pump which depends on the muscles' contractibility of the mouth floor. The ventral layer of a frog's mouth floor is composed of three muscles: 1) the anterior intermandibular muscle, which is the smallest muscle and spans the most rostral parts of the dental bones; 2) the paired *m. intermandibulares*, which are the largest, attach to the dental bones, meet medially, and are connected via a tendon; and (3) the *m. geniohyoideus*, which originates from the hyoid and also meets in the midline of the mouth floor. Contraction of the *m. intermandibulares* and *m. geniohyoideus* lifts the mouth floor upwards, thus narrowing the volume of the buccal cavity. These muscles of the mouth floor produce the main power for elevation of the mouth floor (Walkowiak, 2006).

Muscles are comprised of thousands of myocytes containing myofibrils, which are long chains of sarcomeres, the contractile units of the muscle cells. Striated/skeletal muscle cells have multiple nuclei per cell (Miller *et al.*, 2002) which are called syncytia. Usually, nuclei locate where the syncytium show the most activity, so the nucleus density of the syncytium might be positively related to its activity. The densities of the two major pairs of muscles of the mouth floor of *O. tormota*, *m. intermandibularis* and *m. geniohyoideus*, were higher than other four frogs which phonate at lower frequencies. The higher nucleus densities of the muscles might imply that the mouth floor of *O. tormota* possesses more efficient activities which perform as contractility here.

In the light of the features mentioned above, we know that *O. tormota* has the ability to extrude more air into the lungs by breathing the air in and produce higher pressure difference between the lungs and the buccal cavity by breathing out.

**Better regulation of air stream from glottis:** *Odorrana tormota*, *O. graminea* and *A. wuyiensis* all have thicker muscles on their back of glottises compared to *P. nigromaculatus*, and their contractions make the glottises open and the air get through. Additionally, *O. tormota* possesses more obvious indentions on the contact surfaces of right and left *cartilago gutturalis*. These characteristics can make *O. tormota* control air stream well when the frog communicates by phonation.

**4.4.2 Thinner vocal cords** This study shows that *O. tormota* has the thinnest vocal cords. Driven by certain air stream, the thinnest vocal cords vibrate at the highest

frequency, which is in accord to the truth that *O. tormota* possesses the highest phonation fundamental frequencies in the five species.

**4.4.3 Moderate regulation of amplitudes of the vocal cords** The five species studied have T-shaped vocal cords, of which the lateral parts are connected to the laryngeal skeleton. This connection can regulate the amplitudes of the vocal cords effectively to improve their oscillating frequencies when certain air stream passing by. *Pelophylax nigromaculatus* possesses the loosest connection among the five species. On the contrary, the medial and lateral parts are integrated together for *A. wuyiensis*. So, the three *Odorrana* species including *O. tormota* possess moderate connections. This moderate connection can increase the oscillating frequency of vocal cords by not only avoiding too big amplitudes, but also not encumbering their vibrations.

## 5. Conclusion and Prospects

In this study we probed into the correlations between fundamental frequencies and the phonatory organ features of five frogs including ultrasonic and non-ultrasonic species with sympatric distribution. The results above support that higher contractility of the mouth floor, better regulation of air stream from glottis, thinner vocal cords and moderate regulation of amplitudes of the vocal cords are likely favored by ultrasonic phonation of *O. tormota*. However, we did not test the maximum frequencies' correlations with the phonatory organs because of recording limitation. This also presents a fertile avenue for further research on the relationships between the harmonics of frogs' ultrasound and their special vocal tracts, such as the free open of Eustachian tubes (Gridi-Papp *et al.*, 2008).

**Acknowledgments** We thank Jian LI for his help in collecting specimens, and Yan CAO and Feizhou LEI for their assistance in laboratory work. This study was funded by the National Natural Science Foundation of China (NSFC 30730029, 31071906) and the Main Direction Program of the Knowledge Innovation of Chinese Academy of Sciences (KSCX2-YW-Z-0905, KSCX2-EW-J-22).

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